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Mitochondrial kinases in cell signaling: Facts and perspectives ☆

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ABSTRACT

Phylogenetic studies had shown that evolution of mitochondria occurred in parallel with the maturation of kinases implicated in growth and final size of modern organisms. In the last years, different reports confirmed that MAPKs, Akt, PKA and PKC are present in mitochondria, particularly in the intermembrane space and inner membrane where they meet mitochondrial constitutive upstream activators. Although *a priori* phosphorylation is the apparent aim of translocation, new perspectives indicate that kinase activation depends on redox status as determined by the mitochondrial production of oxygen species. We observed that the degree of mitochondrial oxidation of ERK Cys^{38} and Cys^{214} discriminates the kinase to be phosphorylated and determines translocation to the nuclear compartment and proliferation, or accumulation in mitochondria and arrest. Otherwise, transcriptional gene regulation by Akt depends on Cys^{60} and Cys^{310} oxidation to sulfenic and sulfonic acids. It is concluded that the interactions between kinases and mitochondria control cell signaling pathways and participate in the modulation of cell proliferation and arrest, tissue protection, tumorigenesis and cancer progression.

Contents

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1. Introduction: the world of kinases

An efficient system of signaling cascades has evolved to ensure the ordered development, growth, homeostasis and reproduction of multicellular organisms. This allows cells to respond to environmental stimuli as well as to each other by integrating the numerous extracellular and intercellular cues that they are constantly receiving into a coordinated response. Ever since the discovery nearly 50 years ago that reversible phosphorylation regulates the activity of glycogen phosphorylase, there has been an intense interest in the role of protein phosphorylation in regulating protein function. The completion of the human genome sequence allowed the identification of almost all human protein kinases. The strikingly large size of the kinome constitutes about 2% of all human genes [1]. Protein kinases mediate most of the signal transduction in eukaryotic cells; by modification of substrate activity, the activated protein is translocated from the cell membrane to the nucleus, where gene transcription rate is regulated. However, it is by no means the only pathway that signaling molecules can take. Recent data strongly suggest that, in addition to signaling cascades initiated by hormones or growth factors, reactive oxygen species (ROS) are involved in physiological signaling pathways regulating various cellular functions [2,3] with hydrogen peroxide (H_2O_2) being the main messenger molecule. Mitochondria are suitable as a point of integration for these signaling pathways due to their critical role in cellular metabolism, redox balance, and survival–death mechanisms.

Research on mitochondria has evolved from bioenergetics to biogenesis and is particularly focused on diseases associated with mitochondrial dysfunction. Furthermore, they play also an important role in the regulation of apoptotic cell death through mechanisms which have been conserved through evolution [4]. Although extensive research on Bcl-2 pathways and the release of mitochondrial death mediators have shed a light on survival mechanisms [5,6], little is known about cell communication, in terms of the regulation of transcription of nuclear and mitochondrial genes or protein trafficking to the organelle. Thus, a new era in mitochondrial research has emerged that concerns the role of this organelle in intracellular signaling. This process is prone to have extensive implications in development, aging, and environmental adaptation.

Recently, numerous studies have demonstrated that kinases are translocated to the mitochondria [7,8], where they regulate mitochondrial activities such as phosphorylation of respiratory chain proteins [9,10] and the release of mitochondrial components that ultimately affect the whole cell responses. In addition, mutations of protein kinases and their dysregulation not only play causal roles in human disease, but also open new ways of knowledge to develop agonists and antagonists for use in disease therapy. This review summarizes the evidence on kinase translocation to mitochondria, their differential activation under redox stimuli, the final determination of cellular fate and the current therapies directed against the kinase pathways in cancer and ischemia.

1.1. The MAPK cascade

Mitogen-activated protein kinases (MAPKs) are proline-directed serine/threonine kinases [11] that have been classified into at least five subfamilies; among them, ERK1/2 (extracellular signal regulated protein kinases), JNK1/2 (c-Jun N-terminal kinase), and p38 are the most extensively studied. ERK1/2 is generally activated by growth signals [12,13]; JNK1/2 and p38 respond to oxidative stress, heat shock, ionizing radiation, and UV light [14,15], and are mainly associated with cell cycle arrest and apoptosis. Noteworthy, oxidative stress may be viewed as a potential carcinogen due to the activation of NFκB pathway or by causing DNA mutations [16]. MAPKs are specifically regulated by a MAPK kinase (MAPKK) [17], that is, ERK1/2 is activated by MEK1/2, p38 by MKK3, and JNK1/2 by MKK4, among others (Fig. 1).

The MEK/ERK cascade, is activated in response to protein tyrosine kinase receptors, such as EGF receptor (EGFR) or VEGF receptor (VEGFR) [18]. Growth factor binding induces receptor dimerization and phosphorylation of the cognate receptor by intrinsic tyrosine kinases. Tyrosine phosphorylation of the receptor induces recruitment of proteins that contain SH2 (Src homology 2) domains, including the adaptor protein Grb2. Grb2 is constitutively bound to the Ras activator Sos and is normally localized to the cytosol. This relocation activates Sos, which in turn activates Ras. Ras is a GTPase and hydrolyzes guanosine triphosphate (GTP) to guanosine diphosphate (GDP). When bound to GTP, Ras is able to bind to, and activate, downstream effectors. Mammalian cells contain three Ras isoforms, H-Ras, K-Ras and N-Ras. When GTP-bound, Ras recruits the kinase Raf to the membrane, where it becomes active. There are three known isoforms of Raf, namely A-Raf, B-Raf and C-Raf. Raf catalyzes the phosphorylation and activation of the dual specificity kinases, MAPK/ERK kinases 1 and 2 (MEK1 and MEK2), which in turn activate extracellular regulated kinases, ERK1 and ERK2. Once active, ERKs dimerize and either translocate to the nucleus, where they phosphorylate transcription factors (such as the Ets family), remain in the cytosol, or translocate to multiple cellular compartments to catalyze the phosphorylation of their substrates [19].

There exist four members of the p38 kinase family, namely α, β, γ and δ. These enzymes are activated by cytokines, hormones, G-protein-coupled receptors and stress, such as, heat or osmotic shock [18]. p38 kinases, which are targets of both MEK3 and MEK6, exhibit numerous substrates, including MAPK interacting kinases (Mnk) 1 and Mnk 2, and eukaryotic initiation factor 4e (eIF4e). p38 regulates angiogenesis, cell proliferation, inflammation and cytokine production. A large body of evidence indicates that p38 activity is critical for normal immune and inflammatory responses. p38 is activated in macrophages, neutrophils, and T cells by many extracellular mediators of inflammation and participates in their functional responses, including respiratory burst activity, chemotaxis, granular exocytosis, adherence, and apoptosis, and also mediates T-cell differentiation and apoptosis by regulating gamma interferon production [20]. Besides, it also regulates the immune response by stabilizing specific cellular mRNAs involved in this process.

The JNK family consists of three ubiquitously expressed members, termed JNK1, JNK2 and JNK3 (also known as SAPKγ, SAPKα, and SAPKβ, respectively). Following activation, JNK is translocated to the nucleus where it phosphorylates and up-regulates several transcription factors, including c-Jun, ATF-2, STAT3 and HSF-1 [19]. JNKs exist in 10 or more different spliced forms ubiquitously expressed, although JNK3 is present primarily in the brain. They are involved in the control of apoptosis and the development of multiple cell types in the immune system.

MAPKs are sensitive to redox changes since they are activated in a variety of cellular systems at different H_2O_2 concentrations [21]. We have previously reported that high phosphorylated ERK1/2 content is associated with proliferation and low steady state H_2O_2 ($[H_2O_2]_{ss}$) in proliferating embryonic and tumoral tissues, while tumor arrest

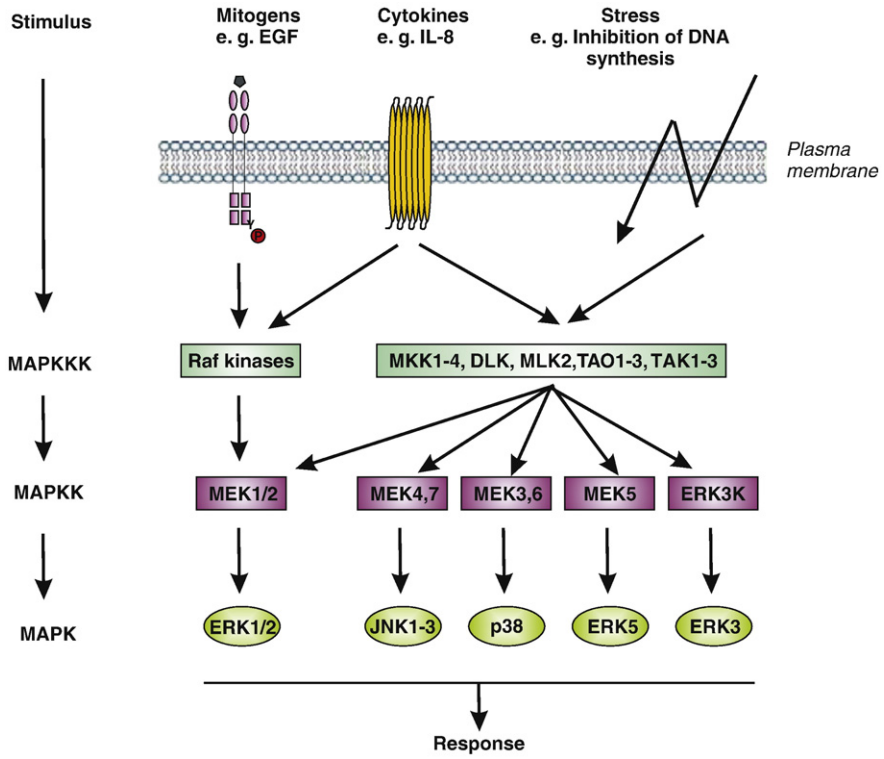

Fig. 1. Mammalian MAPK cascades. The MAPK pathways are stimulated in response to a variety of external cues. Whereas the ERK pathway is commonly activated by growth factors, the JNK and p38 pathways are activated by environmental stress. However, crosstalk occurs, enabling this complex network to control cellular behaviour. Adapted from Raman and Cobb [187]. MAPKKK, MAPK kinase kinase; MAPKK, MAPK kinase; ERK, extracellular regulated kinase; MEK, MAPK/ERK kinase; JNK, c-Jun N-terminal kinase; MEKK, mitogen ERK kinase kinase; DLK, dual leucine zipper-bearing kinase; MLK2, mixed lineage kinase; TAO, thousand-and-one amino acids; TAK, transforming growth factor-beta-activated kinase.

requires high $[H_2O_2]_{ss}$ with predominant p38 and JNK1/2 activation [22,23].

1.2. Akt/protein kinase B

Akt (formerly protein kinase B) is a serine/threonine kinase implicated in the regulation of cell cycle progression, cell death, adhesion, migration, metabolism and tumorigenesis. The ultimate effects of Akt activation are determined by the phosphorylation of its downstream effectors located in the cytoplasm and nucleus, as well as in other cellular compartments. Although the Akt isoforms are ubiquitously expressed in mammalian cells, evidence suggests that the relative isoform expression levels vary between tissues. Akt1 appears to be the mainly expressed isoform in most tissues, while Akt2 is highly enriched in insulin target tissues [24]. Akt1 deficient mice show normal glucose tolerance and insulin-stimulated glucose clearance from blood but display severe growth retardation [25,26]. Cells derived from Akt1 deficient mouse embryos are also more susceptible to proapoptotic stimuli. In contrast to what is observed in Akt1 null mice, disruption of the Akt2 locus results in insulin resistance and a diabetes mellitus-like phenotype [27].

Akt kinases are classically activated by engagement of receptor tyrosine kinases by peptide growth factors and cytokines, as well as oxidative stress and heat shock. Akt activation depends on PtdIns-3,4,5-P_3, and to a lesser extent on PtdIns-3,4,5-P_2, which are products of phosphoinositide 3-kinase [28]. The interaction of PtdIns-3,4,5-P_3 with the Pleckstrin homology (PH) domain of Akt1 favors the binding with their upstream activators and it undergoes phosphorylation at two sites, one in the activation loop (Thr^{308}) and the other in the carboxy-terminal (C-terminal) tail (Ser^{473}). Phosphorylation at Ser^{473} appears to precede and facilitate phosphorylation at Thr^{308} [29]. Akt1 is phosphorylated in Ser^{473} by mTORC2 [30] while the kinase responsible for phosphorylation in Thr^{308} is PI-3K-dependent kinase 1 (PDK1) [31]. More important, the S473D mutant of Akt1 and Akt1 phosphorylated in Ser^{473} by the rictor-mTOR complex are better targets of PDK1 than nonphosphorylated Akt1 [29]. These findings suggest that phosphorylation at Ser^{473} may provide a docking site for PDK1. Once activated, Akt not only phosphorylates and ever-increasing list of substrates in cytosol but also can translocate to the nucleus and mitochondria.

1.3. Protein kinase C

The PKC family of Ser/Thr kinases comprises 10 members in the AGC kinase branch of the kinome. The full-length enzyme is stimulated by products downstream of phospholipase C (PLC)-catalyzed hydrolysis of PIP_2, DAG and Ca^{2+} [32]. All 10 mammalian PKC isoforms contain a highly conserved kinase core at the C-terminal and an amino-terminal autoinhibitory pseudosubstrate peptide, but differ by, and are classified according to, their divergent amino-terminal regulatory regions [33]. Conventional PKC isoforms (cPKC) contain a Ca^{2+}-sensitive C2 domain and two tandem DAG-sensitive C1 domains; novel PKC isoforms (nPKC) contain a Ca^{2+}-insensitive C2 domain and two tandem DAG-sensitive C1 domains; and atypical PKC isoforms contain a single C1 domain that is insensitive to DAG. PKC is maintained in an inactive conformation by binding of the pseudosubstrate sequence to the substrate-binding cavity. Generation of diacylglycerol and Ca^{2+} recruits PKC to the membrane by engaging the C1 and C2 domains on the membrane. This membrane interaction provides the energy to release the pseudosubstrate from the substrate-binding cavity, allowing substrate binding and phosphorylation [34]. However, before such allosteric regulation can occur, PKC must be processed by phosphorylation.

PKC translocation to the plasma membrane generally has been considered the hallmark of activation (and frequently has been used as a measure of PKC isoform activation in cells). However, this simple

model of PKC activation is not sufficient to explain the complex spatiotemporal controls of PKC localization in cells. For example, cPKCs (PKCα, PKCßI, and PKCßII) rapidly/transiently translocate to the plasma membrane via a mechanism that involves PLC-derived DAG accumulation. However, in cells displaying a biphasic DAG response, PKCα and PKCßII (but not PKCßI) are released from the plasma membrane via a regulated process that requires PKC catalytic activity [35]. PKCα/PKCßII then accumulate at a perinuclear site named the "pericentron" as a result of sustained DAG formation through a PLC-independent mechanism involving phospholipase D (PLD); PLD is a membrane-bound enzyme that generates phosphatidic acid (PA; through the hydrolysis of phosphatidylcholine), which is subsequently converted to DAG by PA phosphohydrolase [36].

PKC isoforms also translocate to specialized membrane compartments such as lipid rafts or caveolae [37]. Lipid rafts are sphingolipid-/cholesterol-enriched plasma membrane microdomains that contribute to signal transduction through coalescence into large platforms that concentrate signaling complexes. Caveolae are the sphingolipid/cholesterol-enriched detergent-resistant membranes that form flask-like invaginations of the plasma membrane in cells expressing caveolin. Certain PKCs also accumulate in the Golgi, nucleus or mitochondria.

1.4. Protein kinase A

PKA is the principal target of the intracellular second messenger cAMP. The cAMP/PKA signaling pathway is activated by a number of different receptors that couple to G-proteins upon binding of their respective ligands. Mammalian PKA, like the yeast counterpart, consists of two regulatory subunits (R subunits) which bind cAMP and two catalytic subunits (C subunits) [38]. The complete holoenzyme is not active, although when cAMP binds to the R subunits, the C subunits are able to dissociate as free and active units [39] and can migrate to different cellular compartments [40]. Phosphorylation of nuclear and cytoplasmic substrates mediated by PKA is critical for multiple cell functions, including metabolism, differentiation, synaptic transmission, ion channel activity, growth and development [41]. Although distinct C-PKA subunits have been isolated, the biochemical and functional features of PKA holoenzymes are basically determined by the structure and properties of their R subunits. RIα and RIIα are ubiquitous, whereas RIIβ and RIβ are expressed predominantly in the endocrine, brain, fat and reproductive tissues [42]. In addition to their typical expression and distribution, R subunits vary in their regulatory and biochemical properties. Phosphorylation at two sites at the C-terminal, Thr^{197} and Thr^{338}, is important for catalysis and stabilization. In contrast with other AGC kinases, phosphorylation of the C-subunit occurs before assembly into an inactive holoenzyme complex. Structural and biochemical studies have shown that phosphorylation at Ser^{338} stabilizes the kinase core and confers resistance to phosphatases [43].

PKAII is concentrated in particulate membranes and cellular organelles through interactions with a family of A-Kinase-Anchor-Proteins (AKAPs). Although the preferred ligand is RII/PKAII, several AKAPs also bind RI/PKAI [44]. Each AKAP includes a targeting domain that binds it to a specific cytoskeletal or membrane element and a PKA binding motif that anchors PKAII by binding RII subunits. AKAPs enhance the efficiency of cAMP signal-transducing pathways by localizing PKA near cAMP generation sites or targets [45].

2. Mitochondria in reactive oxygen species metabolism and signaling

2.1. Characteristics of the mitochondrial ROS-producing system

For many years, mitochondrial respiration was thought to follow an "all or nothing" paradigm supporting the notion that in the normal O_2 concentration range, respiration is mainly controlled by tissue demands. However, nitric oxide (NO) produced by cytosol or mitochondrial Nitric Oxide Synthases (mtNOS) adapts respiration to different physiological conditions and increases the mitochondrial production of reactive oxygen species (ROS) that contributes to NO clearance. In the physiological condition, 3–5% of O_2 is univalently reduced in normal respiration [46]. Formerly considered toxic bystanders of electron transfer reactions in mitochondria, superoxide anion ($O_2^{\cdot-}$) and its dismutation product H_2O_2 are now recognized as gene modulators throughout oxidation of reactive groups in transcription factors, kinases, and phosphatases. Electron transfer through mitochondrial complexes I–IV is linked to proton pumping across the inner membrane creating a proton electrochemical gradient between the intermembrane space and the matrix. This gradient is dissipated by the reentry of protons through ATPase channels that couple ATP synthesis to the electron transfer activity. From a classic perspective, it is accepted that the rate of this process is regulated by O_2 and substrate availability as well as ADP/ATP ratio in response to cell demands. In the last few years, significant modulatory effects of NO resulted from its high-affinity binding to cytochrome oxidase, the final electron acceptor of electron transfer chain [47].

In addition, mitochondria produce oxygen active species by auto-oxidation of ubisemiquinone, a transitional intermediary redox state of membrane ubiquinol. The ratio of O_2 utilized undergoes one-electron reduction by ubisemiquinone, forming $O_2^{\cdot-}$ which is dismutated by mitochondrial superoxide dismutase (Mn-SOD) to H_2O_2 that freely diffuses to cytosol [48]; besides, mitochondrial $O_2^{\cdot-}$ can be driven to cytosol through voltage-dependent anion channels [49]. The ubisemiquinone pool and $O_2^{\cdot-}$ production rate are increased by utilization of specific compounds, such as antimycin, which blocks electron flow between cytochromes *b* and *c* [48]. Besides the reversible inhibition of cytochrome oxidase, NO induces inhibitory effects on the *b*–*c*1 region at complex III, leading to direct ubiquinol oxidation [50,51].

Considering that NO metabolism regulates mitochondrial O_2 uptake and $O_2^{\cdot-}/H_2O_2$, and its effects on gene expression and cell signaling, it is surmised that mitochondrial NO plays a significant role in the modulation of life processes.

2.2. Mitochondria signaling in cell proliferation and apoptosis

Over the past decade, several investigators reported that H_2O_2 and NO regulate cell proliferation [52,53]. Most of the cells in adult tissues remain in a quiescent state and reenter the cell cycle and continue proliferation only in response to tissue injury or when replacing adjacent cells [54]. It is remarkable that the cell cycle transition from G0 to G1 is not regulated by cyclin-dependent kinases, but rather by redox-sensitive cell signaling and gene expression cascades [53]. In this context, H_2O_2 and NO levels determine cell fate by triggering proliferation, arrest or apoptosis.

The antiproliferative effects of NO have been demonstrated in a variety of cell types from normal tissues and diverse tumors [55,56]. Upon exposure to NO from different sources either NOS or NO-donors, cell stop growth at G1 or G2 phase, or show a delay in S phase progression. Up-regulation of endogenous NO production, by L-arginine supplementation or by expression of the inducible Nitric Oxide Synthase (iNOS), inhibited proliferation of lymphocytes, vascular smooth muscle cells and pancreatic tumor cells [57]. Conversely, the treatment of hematopoietic progenitor cells with a NO scavenger increased cell proliferation [58].

The action of NO on the cell cycle elicits a series of molecular events. A well characterized effect is the up-regulation of the cyclin-dependent kinase inhibitor $p21^{Cip1/Waf1}$ [59] in a process mediated by MAPK. Another remarkable action of NO on cell cycle is the regulation of cyclin expression. Exogenous NO decreases the synthesis of cyclin D1 but not of cyclin E in breast cancer cells and this down-regulation

is accomplished without an increase in the degradation of Cdk4/6 regulatory protein [60].

Noteworthy are the effects of NO and H_2O_2 outcome in the modulation of MAPKs and cyclin D1. ERK stimulates cell proliferation and induction of active cyclin D1 by numerous mechanisms including the enhancement of AP-1 activity. It has been stated that NO induces a gradual elevation of intracellular [Ca^{2+}] that leads to activation of ERK and enhances cell division. The functional blockade of Ca^{2+} and the inhibition of calmodulin prevent ERK activation and antagonize the mitogenic effect of NO [61]. On the other hand, p38 translationally down-regulates cyclin D1. This effect is due to phosphorylation of cyclin D1 at Thr^{286}, which leads to the ubiquitination of the enzyme. Similarly, NO activates p38 and suppresses proliferation through the activation of JAK2-STAT5 and cyclin D1/cdk4 [62].

Hydrogen peroxide is considered an intracellular signal for cell growth and transformation. ERK1/2 translocates into the mitochondria during brain development [21] and ERK activation is a H_2O_2-dependent process. *In vitro*, ERK1/2 activation in mitochondria was maximal at 1 μM H_2O_2, an effect also observed in embryonic hepatoblasts and isolated postnatal P2 hepatocytes, while a decreased phosphorylation concomitant with p38 MAPK activation was observed in the quiescent adult cells (Fig. 2A) [22]. The regulation of MAPKs cascades was related to the modulation of mtNOS in the sequence of proliferating to quiescent cell stages. Proliferating phenotypes are characterized by low levels of mtNOS expression and activity, with a resulting NO-dependent $[H_2O_2]_{ss}$ yield of 10^{-11} to 10^{-12} M, and high cyclin D1 expression associated with high ERK1/2 and low p38 activities. In contrast, quiescent phenotypes presented an opposite pattern with NO-mediated H_2O_2 levels of 10^{-9} M. In accord, increases of mtNOS and H_2O_2 steady state concentration are parallel during rat brain and cerebellum development at the phase of synaptic plasticity [63].

Different effects of H_2O_2 are distinguished in transformed cells; increased proliferation in tumor lung cells P07 and mammary MM3 cell lines was observed at 1 μM H_2O_2, whereas cells became arrested without apoptosis at 50 μM H_2O_2 [23]. It is noteworthy that mtNOS expression is reduced in some tumor cell lines like M3 and MM3 mammary tumors while in others like P07 lung tumors it is high but with an mtNOS activity consistently lower in tumor cells than in normal tissues. Accordingly, mitochondrial H_2O_2 production is significantly lower in tumor cells compared to normal mitochondria. In this context, a low functional level of oxidative phosphorylation, decreased mtNOS, and low NO-dependent H_2O_2 production represent a general situation common to the active growth of tumors and of embryonic tissues (Fig. 2B and C).

The process of programmed cell death or apoptosis is executed through two different pathways, known as the extrinsic and the intrinsic pathways. The extrinsic pathway involves binding of TNF-α and Fas ligand to membrane receptors leading to caspase-8 activation, while the intrinsic pathway involves mitochondrial oxidative stress and damage and mitochondrial cytochrome *c* release. Released cytochrome *c* triggers the assembly of the apoptosome complex with apoptotic protease-activating factor-1 (Apaf-1) and procaspase-9 which induces activation of caspase-9. Both pathways converge on caspase-3 activation, resulting in nuclear degradation and cellular morphological changes [64].

It has been stated that NO directly induces cytochrome *c* release from the mitochondria through mitochondrial potential loss [64] or by tyrosine nitration of cytochrome *c* [65]. High concentrations of NO and peroxynitrite ($ONOO^-$) were reported to cause DNA damage and

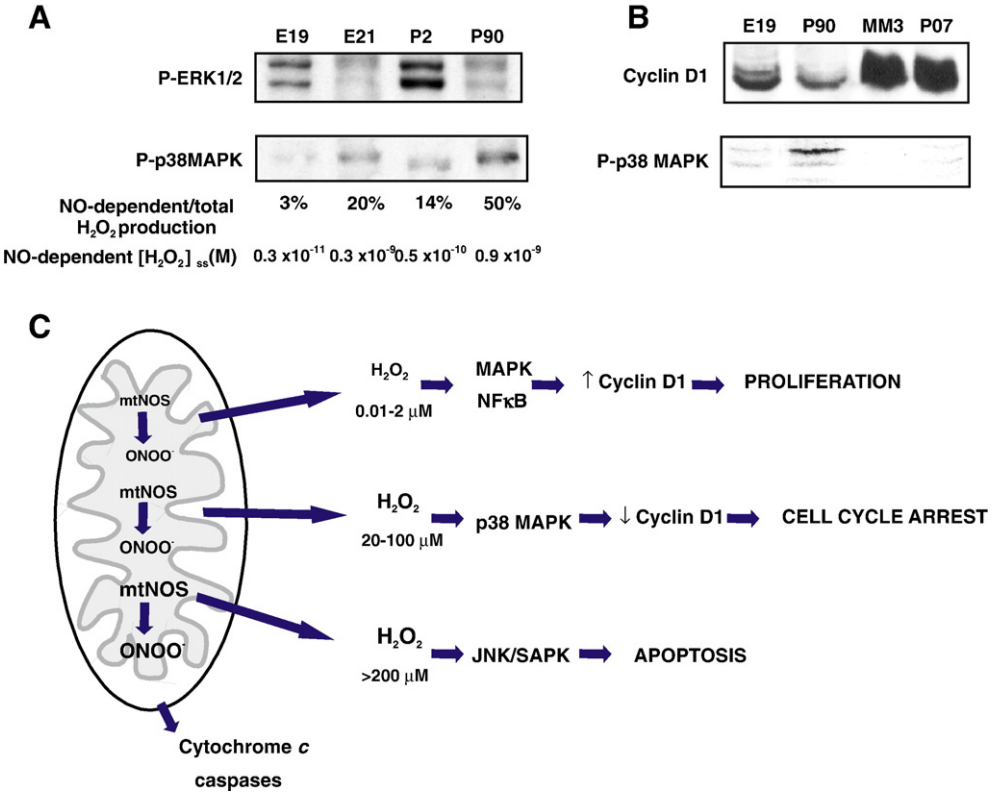

Fig. 2. (A) Modulation of cell signaling in the developing rat liver. Western blots of liver homogenates with antibodies directed to phosphorylated MAPKs: ERK1/2 and p38. Comparative H_2O_2 steady state concentration measurements and calculations from embryonic and adult mitochondria are included. (B) Reciprocal levels of cyclin D1 and p38 MAPK activity in tumoral cell lines and adult and proliferating liver. E19 and E21 represent liver samples harvested from embryos at 19 or 21 days of development while P2 and P90 from postnatal days 2 or 90. MM3 and P07 correspond to tumoral cell lines spontaneously arisen from mammary and lung adenocarcinomas respectively. (C) Redox modulation of cell signaling. Scheme of the transition mechanism of proliferating to quiescent/apoptotic stages depending on the redox status. Inside the scheme, comparative mtNOS activity and H_2O_2 concentration drive signaling from proliferation to apoptosis.

lead to p53-mediated growth arrest and apoptosis in tumor cells [66]. Nuclear factor-κB (NFκB) plays a protective role against apoptosis through the up-regulation of genes encoding antiapoptotic proteins [67]. Nitric oxide inhibits NFκB activation by inducing the expression of the NFκB inhibitor IκBα and by stabilization of the NFκB/IκBα complex [68]. In contrast to NO, oxidative stress activates IκB kinase (IKK) which leads to the phosphorylation of IκBα and activation of NFκB. The activation of IKK and phosphorylation of IκBα is blocked by antioxidants and NO [67]. Inappropriate activation of NFκB has been linked to inflammatory events associated with autoimmune arthritis, asthma, septic shock, lung fibrosis, glomerulonephritis, atherosclerosis, and acquired immunodeficiency syndrome. In contrast, complete and persistent inhibition of NFκB has been linked to apoptosis, inappropriate immune cell development, and delayed cell growth.

Although NO promotes apoptosis in some cells, it also displays antiapoptotic properties in other cell types. It has been claimed that the antiapoptotic mechanism involves the gene transcription of protective proteins, such as heat shock proteins, hemeoxygenase and cyclooxygenase-2 and the direct inhibition of the apoptotic activators of proteases of the caspase family by S-nitrosylation of the cysteine thiol group in the catalytic site in a cell specific way [69].

3. Redox regulation of kinases

3.1. Mechanisms of protein modification by oxidants

Besides its role in stress response to oxidative damage, there is increasing evidence that redox signaling is a part of normal metabolism in non-stressed cells. Endogenously generated oxidants, such as H_2O_2 and NO, can act as second messengers, modulating the activities of molecules involved in key cellular processes, including phosphorylative cascades [70]. Reactive oxidants can integrate into cellular signal transduction pathways through oxidation and reduction of thiol proteins, since cysteine residues are sensitive to oxidation, and changes in enzymatic activity or binding characteristics due to oxidation provide a mechanism for signal transmission [71].

There are two general proposed mechanisms for redox regulation of proteins, and both require that target proteins be transiently oxidized to enable transmission of the signal and then, enzymatically reduced to their basal oxidation state [72]. The first mechanism is given by direct action of the oxidant on key cysteine residues of highly sensitive proteins. These residues are suitable for ROS oxidation due to their low pK_a, which is defined by the molecular environment (i.e., the presence of neighboring positive amino acids that helps ionization), although it is likely that there are other determinants for ROS reactivity. The second mechanism for redox regulation is through an interaction with very reactive thiol proteins that act as sensors of redox state, such as thioredoxins. Once these sensors become oxidized, they facilitate the oxidation of the target protein through selective protein–protein interactions and thiol exchange.

3.2. Modulation of protein kinases by redox state

First evidence for redox modulation of kinase pathways came from studies on protein tyrosine phosphatases (PTPs), key regulators of protein tyrosine kinases (PTKs). These enzymes react rapidly, and become inactivated, when exposed to low micromolar concentrations of H_2O_2, which specifically oxidizes the essential catalytic cysteine residue to a sulfenic acid (Cys-SOH) intermediate, and they could be fully reactivated with glutathione [73]. The cysteine-sulfenic intermediate forms a mixed disulfide that not only inactivates the enzyme, but also protects it from irreversible extra oxidation. In this sense, it has been observed that a number of PTKs, which are at the top of most of signaling cascades, are "primed" for activation through cysteine oxidation, either by inducing conformational changes that make the catalytic site more accessible (like in the case of insulin receptor kinase, IRK) or even by promoting dimerization that results in autophosphorylation (i.e., c-Ret — a receptor tyrosine kinase) [74].

Serine/threonine kinases are also regulated by ROS and other oxidants. For example, the N-terminal regulatory domain of PKCs contains zinc-binding, cysteine-rich motifs that are readily oxidized by peroxide, compromising its autoinhibitory function and, consequently, stimulating cellular PKC activity. On the other hand, the C-terminal catalytic domain contains several reactive cysteines that are targets for various chemopreventive antioxidants, so that modification of these cysteines decreases cellular PKC activity [75]. Another example for redox regulation is the switching of PKA. PKA free C-subunit can be inactivated by oxidation of a highly reactive cysteine, located in the activation loop, which is capable of forming a mixed disulfide with glutathione or an internal disulfide with another cysteine located in the C terminus. In this conformation, dephosphorylation is favored, while preferential re-phosphorylation only occurs after reduction of the kinase, thus creating a redox cycle whereby the kinase phosphorylation state may be regulated [76,77].

Increasing evidence shows that MAPK pathways are redox regulated at different levels [78]. Recently we showed that redox state modulates the mitochondrial interaction of MAPKs to MAPKKs by oxidation to sulfinic and sulfonic acids of conserved cysteine domains involved in the interaction of the MAPKs with their upstream kinases. Oxidation to sulfinic and sulfonic acids is generally thought to be irreversible, and it has sense in a tumoral context, where constitutive activity of certain pro-proliferative kinases – and inhibition of proapoptotic ones – is required. Even more, it was found that selective kinase activation is based upon a differential sensitivity to certain oxidants, since ERK2 was oxidized and activated at low H_2O_2 concentrations but its activity was reduced and no oxidation was detected at high H_2O_2 levels, and the opposite effects were seen with the proapoptotic JNK1/2 and p38 kinases [79].

Cysteine reactivity of most proteins could be not enough to become oxidized at physiological levels of ROS [70,72]. The best characterized model describing an indirect mechanism for redox kinase activation is the system Trx1/ASK1. Besides its role as a general protein disulfide reductant that contributes to balance the intracellular redox state, thioredoxins (Trx) are being postulated as signaling intermediates that sense the redox status and transmit their information to signaling molecules such as protein kinases [80]. Trx was identified as a binding protein of ASK1, a MAP3K of the MKK4/JNK and MKK3/p38 MAPK signaling cascades. If Trx is reduced, it is bound to the N-terminal region of ASK1 and inhibits its kinase activity. When become oxidized, the two cysteine residues of its redox active site form an intramolecular disulfide bond and Trx dissociates from the ASK1, which gains kinase activity by autophosphorylation of two threonine residues at the activation loop [81].

3.3. Cellular consequences of protein-thiol modifications

As summarized, oxidation of key protein-thiol groups induces conformational changes on protein kinases that lead to activation or inhibition of kinase activity by direct alteration of the active site, changes in phosphatases activity, or binding to upstream kinases and regulatory proteins. Each of these changes has different consequences in cell fate, depending on the pathways implicated and the physiological context [82]. For instance, our previous reports indicate that high phosphorylated ERK1/2 content was associated with proliferation and low $[H_2O_2]_{ss}$ in proliferating embryonic and tumoral tissues, while normal differentiation and tumor arrest required high $[H_2O_2]_{ss}$ with predominant p38 and JNK1/2 activation [22,23].

Cellular compartmentalization is another biological feature that can influence differential cellular responses to redox switches [83]. Our recent findings showed that low $[H_2O_2]_{ss}$ causes GFP-hERK2 entrance to the mitochondria and subsequent translocation to the nuclei, favoring the proliferative state. In contrast, GFP-hJNK1 enters

the organelle only after high redox condition, leading to arrest or apoptosis. In spite of low oxidant stimulation, ERK2 non-oxidable mutants (C38A and C214A) are retained in mitochondria in detriment of nuclear entrance. This biochemical mechanism determines MAPK differential activation and traffic to nuclei and ultimately, sustains the phenotype of LP07 tumor cells [79].

4. Kinases in mitochondria

4.1. MAPKs

Many studies using pharmacologic inhibitors of MEK have indicated that ERK can modulate mitochondrial functions, particularly those associated with cell death. For instance, ERK signaling appears to promote mitochondrial ATP synthase function in glucose-deprived astrocytes [84], to maintain mitochondrial membrane potential and prevent cytochrome *c* release [85], and to inactivate the proapoptotic protein BAD [23]. Mitochondrial fractions of normal rat brain homogenates show 10-fold lower levels of ERK1/2 than those observed in crude homogenates [86]. However, the presence of a mitochondrial pool of ERK1/2 in normal as well as stressed tissues supports a potential physiological role for ERK in mitochondrial regulation. Our group has previously established the presence of ERK1/2, p38 and JNK1/2 within the mitochondrion (Fig. 3A and B) [21,79]. ERK1/2 translocation to brain mitochondria follows a developmental pattern which is maximal between E19-P2 stages and afterwards declines at P3, just before maximal translocation to nucleus, and up to adulthood. These results suggest that developmental mitochondrial activation of ERK1/2 cascade contributes to its nuclear translocation effects, providing information about mitochondrial energetic and redox status to the proliferating/differentiating nuclear pathways.

Interestingly, both ultrastructural and biochemical subfractionation studies showed ERK localized within the mitochondrion in association with the outer membrane/intermembrane space fraction [21]. Consequently, it is clear that ERK is present in an ideal location for modulating mitochondrial death mediators and respiratory or metabolic processes.

Many studies point out a role for p38 MAPK signaling in regulating cell death events, including translocation of Bax from cytosolic to mitochondrial compartments [87], caspase-independent potassium efflux [88], and transcriptional regulation of TR3, a steroid receptor-like protein that translocates from the nucleus to the mitochondria to initiate the intrinsic pathway of apoptosis [89]. Involvement of MAPKs in regulating the mitochondrial death pathway is particularly well established for JNK, not only through the activation of intermediates like Bax [90], but also by many subcellular fractionation studies showing localization of activated JNK to the mitochondria. In addition,

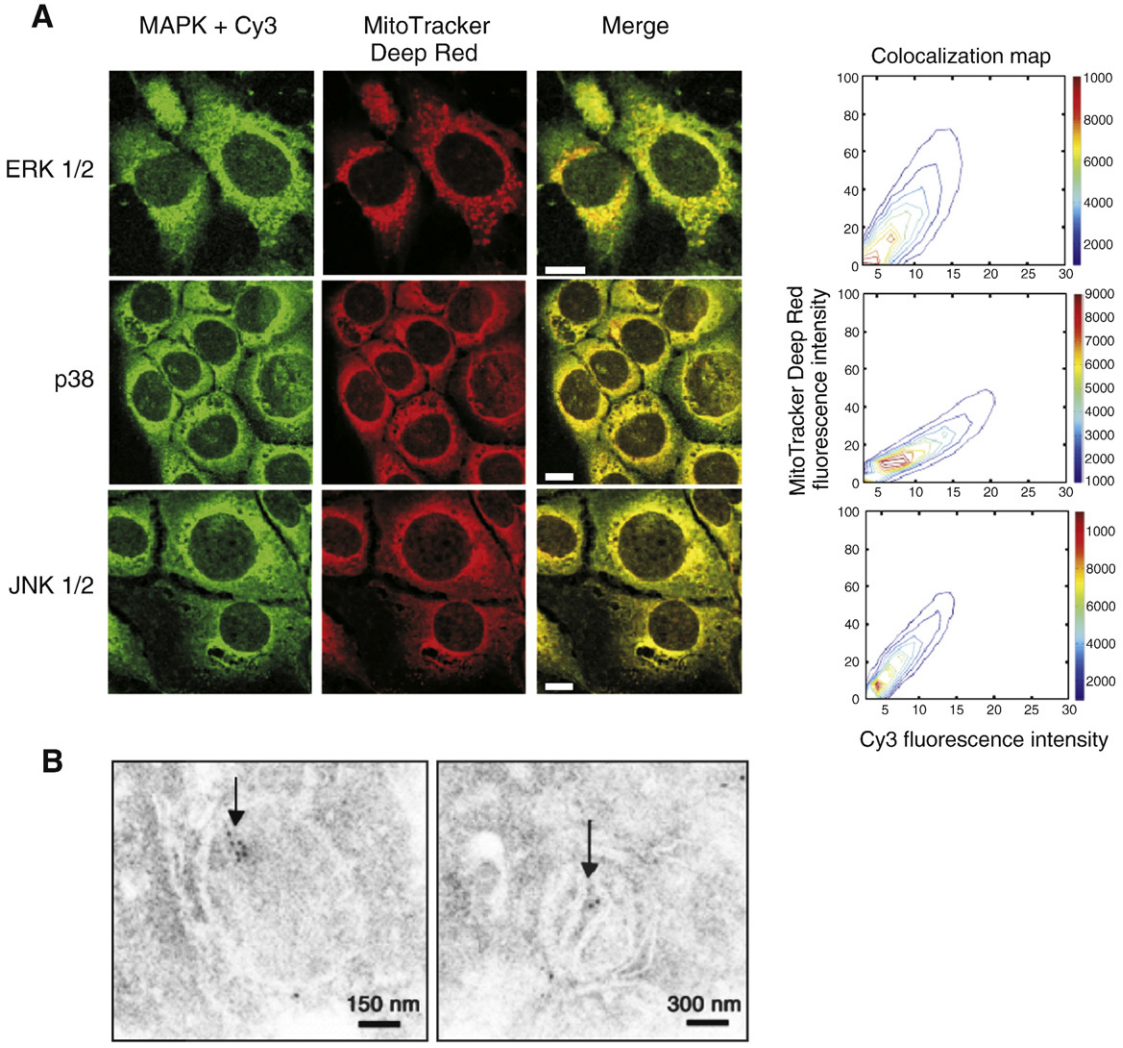

Fig. 3. MAPKs in mitochondria. (A) P07 cells were stained with MitoTracker Deep Red, fixed and immune stained with anti ERK1/2, JNK1/2 and p38 primary antibodies and secondary antibodies conjugated with Cy3, and analyzed in an Olympus FV1000 confocal microscope. Pixel frequency map displayed on the right. Bar = 10 μm. (B) ERK1/2 was detected in P07 mitochondria by immune labelling and transmission electron microscopy.

recent detection of mitochondrially targeted scaffold proteins lends compelling evidence for the biological relevance of mitochondrial JNK. For example, Sab (SH3BP5) which is a JNK-binding protein that co-localizes with mitochondria [91] may serve a function analogous to that of certain AKAPs in the localization of kinase activity to the mitochondria. The effects of JNK on the mitochondria often involve stimulation of apoptosis. Treatment of isolated rat brain mitochondria with active JNK causes the inhibition of antiapoptotic Bcl-2 and Bcl-xL, promoting the release of cytochrome *c* and a decrease in Dcm [92].

To understand the mechanisms of redox modulation by MAPKs, some studies have been focused on the oxidative inhibition of phosphatases (MKP) [93] and on the interaction of MAPKKs with antioxidant proteins, such as thioredoxin [94]. However, we have recently shown that the redox state modulates the mitochondrial interaction of MAPKs to MAPKKs by oxidation of conserved cysteine domains of MAPKs to sulfinic and sulfonic acids. Specifically, redox state determines MAPKs subcellular redistribution and subsequently sustains the phenotype of LP07 tumor cells [79]. In this way, modulation of MAPK interaction with their upstream kinases relies on the oxidation of cysteines immersed in conserved domains of MAPKs. A remarkable finding is that selective kinase activation is based on a differential sensitivity to oxidants, particularly H_2O_2.

4.2. Akt

Akt exerts a major role in apoptosis due to its networking with the mitochondria in the regulation and inter-connection of metabolic pathways and cell survival. Akt is able to directly or indirectly modulate apoptosis [95]. The direct effects are linked to phosphorylation events or interactions with cell death actors, whereas the indirect regulation of apoptosis is mediated rather through transcriptional responses to apoptotic stimuli. In both cases, Akt is at the crossroads of several mitochondria-mediated cell death pathways and thus, an important target for cancer therapy [96]. Bad, as the first PKB target involved in apoptosis (identified in 1997) [97] is a proapoptotic member of the Bcl-2 family of proteins. By phosphorylating the proapoptotic factor Bad, Akt prevents the release of cytochrome *c* from the mitochondria [98]. In addition, the caspase cascade is further inhibited by Akt phosphorylation of procaspase 9 [99]. At the same time, Akt deliver antiapoptotic signals via positive and negative transcriptional mechanisms preventing transcription of proapoptotic genes such as Fas ligand, Bim, Trail and Tradd. Akt kinases are also involved in intermediary metabolism, in particular glucose metabolism. Its substrate, glycogen synthase kinase 3ß (GSK3), is inactivated by phosphorylation, leading to an increased glycogen synthesis. Following insulin stimulation, glucose transport is increased by Akt phosphorylation and membrane translocation of the glucose transporters GLUT1 and GLUT4 [100]. Our group has recently demonstrated that at a precise concentration, insulin increases P-Akt2 that translocates to the mitochondria and determines *in situ* phosphorylation and substantial cooperative mtNOS activation, high NO, and a lowering of mitochondrial oxygen uptake and resting metabolic rate. Thus, insulin signaling activates Akt2, that not only stimulates the GLUT4 recycling pathway but also increases NOS activity in the organelles in an attempt to restrain mitochondria, thus favoring the replenishment of glycogen and fat energy stores [101].

It has been reported that insulin-like growth factor 1 results in rapid translocation of P-Akt into mitochondrial subcellular fractions of neuroblastoma and human embryonic kidney cells [102]. Activated mitochondrial Akt resulted in phosphorylation and further inhibition of the β-subunit of ATP synthase and of GSK3ß. GSK3ß phosphorylates and inhibits mitochondrial pyruvate dehydrogenase activity and promotes apoptosis [103]. The mitochondrial import and export mechanisms for Akt have yet to be investigated. However, the close correlation between phosphorylation of cytosolic Akt, the accumulation in mitochondria and the rapid phosphorylation of mitochondrial GSK3ß suggests that these events are linked, possibly through an Akt phosphorylation-dependent uptake or retention mechanism.

4.3. PKC

Nearly 15 years ago, the α and β isoforms of PKC were detected in a subset of mitochondria in carp retinal Müller cells [104]. These immunoelectron microscopy studies showed that the kinase was localized in the inner membrane. Since that time, large emerging evidence indicates that PKC isoforms play a direct role in regulating mitochondrial function.

Two isoforms of a novel type of PKC, PKCδ and PKCε, show opposite effects on apoptosis. For example, activation of PKCδ induces and/or enhances the apoptotic events that occur during ischemia–reperfusion and malignant progression of cancer cells, whereas activation of PKCε inhibits and/or reduces these events [105].

Renal proximal tubular cells react to oxidative stress by trafficking activated PKCε to the mitochondria, which inhibits the electron transport chain, ATP production, and Na^+ transport, possibly in part through direct phosphorylation of Na^+-K^+-ATPase [106]. Overexpression of wild type PKCε in MCF-7 cells inhibits activation of caspases 8 and 9 and decreases tumor necrosis factor-induced mitochondrial depolarization, which leads to the release of mitochondrial cytochrome *c* and cell death induced by TRAIL [107]. The level of the antiapoptotic protein Bcl-2 increased, whereas that of the apoptotic protein Bid decreased by PKCε at both the protein and mRNA levels. Furthermore, PKCε depletion or overexpression of a dominant negative PKCε is associated with a decrease in Bcl-2 protein levels. These findings offer clear evidence that PKCε mediates its antiapoptotic effect via the mitochondria by regulating both the activities of proapoptotic and antiapoptotic proteins as well as the translocation of these proteins to mitochondria [108].

When neoplastic cells are treated with phorbol esters, H_2O_2, or anticancer agents such as cisplatin or etoposide, PKCδ enters the mitochondria, where it elicits the release of cytochrome *c* and the consequent induction of apoptosis [109]. Similar results are shown in keratinocytes, in which exposure to UV radiation prompts PKCδ activation and mitochondrial translocation, followed by disruption of the mitochondrial membrane potential, caspase release, and apoptosis [110].

4.4. PKA

Early studies identified cAMP-dependent kinase activity in purified preparations of mitochondria from a guinea pig and rat liver suggesting the presence of mitochondrial PKA [111]. Over the last years, it has been demonstrated that PKA plays a critical role in mammalian mitochondrial physiology suggesting that locally activated PKA can efficiently phosphorylate mitochondrial substrates and thus, modulate their function. Numerous mitochondrial proteins are substrates of PKA, including the nuclear-encoded 18-kDa subunit of complex I (NDUFS4), the proapoptotic Bad protein and steroidogenic acute regulatory (StAR) protein. Phosphorylation of NDUFS4 by PKA boosts the activity of mitochondrial respiratory complexes [112]. This would increase ATP synthesis in response to conditions that induce cAMP signal transduction.

Activation of PKA by extracellular ligands or cAMP analogs inhibits apoptosis since PKA phosphorylates and inactivates Bad. Unphosphorylated Bad binds and inactivates antiapoptotic Bcl-2 homologues. This causes the release of cytochrome *c* from mitochondria and the resultant activation of the apoptotic pathway. Phosphorylation by PKA disrupts Bad association with Bcl-2 and inhibits apoptosis. cAMP signaling to mitochondria depends crucially on the localization of PKA to the outer mitochondrial membrane (OMM). Delocalization of PKA from mitochondria induced by dominant negative AKAP121 mutants reduces phosphorylation of Bad and elicits apoptosis [113]. On the

contrary, overexpression of AKAP121 increases Bad phosphorylation and protects cells against proapoptotic stimuli [114].

Transport of cholesterol from cytosol to mitochondria is a highly regulated process that is stimulated by PKA-dependent phosphorylation and activation of StAR. Temporal mitochondrial ERK1/2 activation is required for PKA-mediated steroidogenesis in the Leydig-transformed MA-10 cell line [115]. PKA activity leads to the phosphorylation of a constitutive mitochondrial MEK1/2 pool. In addition, mitochondrial maximal steroidogenesis occurs as a result of the mutual action of StAR, active ERK1/2 and PKA. Interestingly, it has been demonstrated that StAR is a novel substrate of ERK1/2, and that mitochondrial ERK1/2 is part of a multimeric protein kinase complex that regulates cholesterol transport [115].

AKAP121/149 has an RNA binding motif, a KH domain. Ginsberg et al. [116] have shown that, besides anchoring PKA, AKAP121 localizes at least two mRNAs of nuclear-encoded mitochondrial proteins. Experiments in HeLa cells point out that a complex of mRNA and PKA is targeted to mitochondria by AKAP121. Purified AKAP121 KH domain binds the 3′UTRs of transcripts that encode the Fo-f subunit of mitochondrial ATP synthase and Mn-SOD. Binding entails a structural motif in the Fo-f mRNA 3′UTR, and is stimulated by PKA phosphorylation of the KH domain [116].

Moreover, Alto et al. [117] have demonstrated that Rab32, a member of the Ras superfamily of small molecular weight G-proteins, interacts directly with type II regulatory subunit of PKA functioning as an AKAP. Rab32 and a proportion of the cellular PKA pool are associated with mitochondria. Transient transfection of a GTP binding-deficient mutant of Rab32 promotes aberrant accumulation of elongated mitochondria at the microtubule organizing center. This implicates Rab32 as a key in synchronization of mitochondrial fission. Thus, Rab32 is a dual function protein that participates in both mitochondrial anchoring of PKA and mitochondrial dynamics [117].

5. Deregulation of signaling pathways in disease

5.1. Cancer

The role of the MAPK signaling pathway in cancer has been the focus of intense research. Ras genes encoded by rat sarcoma virus, *v-H-Ras* and *v-K-Ras*, were among the first oncogenes discovered [118]. Mutant Ras proteins are constitutively GTP-bound, and thus constitutively active. Ras is the most common mutated oncogene in human neoplasms, with 30% of all cancers harbouring a Ras mutation (Table 1) [119]. In addition to Ras, other proteins in the MAPK cascade contribute to carcinogenesis. The B-Raf mutation, V600E, has been identified in about 60% of patients with human melanoma and is the most common mutation in such cancers [120]. The V600E mutation results in highly active B-Raf kinase, which becomes independent of the GTP/GDP-bound state of Ras. Therefore, the Raf/MEK/ERK pathway is hyperactive. Increased ERK phosphorylation and expression has been found in pancreatic cancer [121], and increased ERK phosphorylation correlates with tumor progression in prostate cancer [122]. Augmented MEK phosphorylation has been recognized in colon cancer [123] and in 74% of myeloblasts in acute myelogenous leukaemia [124]. Finally, increased MAPK activity has also been shown in breast cancer [125]. Treatment of breast cancer cells with estradiol activates MAPKs directly and indirectly through increased production of TGFα, IGF-1 and IGFR [126].

Table 1
Human tumors exhibiting activated RAS-MAPK signaling.

Tumor type	Pathway mutations
Colon	KRAS (45%), BRAF (12%), EGFR (30%)
Pancreatic	KRAS (90%), EGFR (30–50%)
Ovarian	BRAF (30%)
Melanoma	NRAS (15%), BRAF (66%)
Non-small-cell lung	KRAS (35%), EGFR (14–90%)
Papillary thyroid	HRAS, KRAS and NRAS (60%); BRAF (35–70%)
Lung	EGFR (40–80%)
Glioblastoma	EGFR (20%)
ALL, AML	NRAS (30%)

ALL: acute lymphocytic leukaemia; AML: acute myeloid leukaemia.

Proteins that alter the subcellular localization of ERK1/2 have the capacity to impact cancer disease. For example, the cytoplasmic retention of ERK1/2 occurs in a fraction of breast cancer patients and is suggested to favor long-term survival of these patients [127]. Expression of Mxi2, a p38 MAPK splice form, increases the concentration of ERK1/2 in the nucleus. Mxi2 is overexpressed in certain renal cancers and its effect on ERK1/2 localization may contribute to disease [128].

After the p53 pathway, the PI3K–PTEN–PKB/Akt signaling pathway is one of the most mutated pathways associated with cancer [129]. This pathway is activated in many tumors as a result of upstream mutations in PI3K [130] or PTEN (phosphatase and tensin homologue mutated in multiple advanced cancer) [131], or of the amplification/overexpression/mutation of PKB isoforms themselves [132]. Consequently, the constitutive activation of PKB contributes greatly to aberrant cell cycle regulation, a hallmark of many cancers, resulting in uncontrolled cell proliferation and suppressed apoptotic pathways [133]. It was nearly two decades since the initial description of AKT by Staal [134] before polymorphisms and somatic mutations were identified that directly link mutations in Akt isoforms to human cancer. Large scale sequencing efforts have identified germline single nucleotide polymorphisms and somatic mutations in AKT2 and AKT3 [135]. Carpten et al. [136] have described an activating, although rare, mutation in the pleckstrin homology domain of AKT1 in breast, colorectal and ovarian clinical cancer specimens. Others have since confirmed these findings in various cancers [137]. Specifically, a G>A mutation at nucleotide 49 results in a lysine substitution for glutamic acid at amino acid 17 [AKT1(E17K)] that results in a mutant Akt1 protein with increased plasma membrane recruitment. In culture, cells carrying the mutant allele exhibit a transformed phenotype. Retroviral transduction into Eμ-Myc transgenic mice induces leukaemia similar to myristoylated Akt1 that recapitulates the GAG-Akt fusion protein transduced by AKT8 [136]. Activation of a cytoplasmic kinase by means of a PH domain mutation has previously been reported for Bruton's tyrosine kinase (Btk) [138]. Still, the identification of such a mutation in Akt is an important confirmation for a direct involvement of Akt in cancer which was previously lacking supporting genetic evidence. Thus, both direct changes in Akt and also indirect changes in modifiers of Akt activity increase pathway strength and drive inherited and somatic tumors in humans [139].

Normal and neoplastic keratinocytes undergo apoptosis when PKCδ is overexpressed and is further translocated to the mitochondria, altering its membrane potential [140]. In fact, a lack of PKCδ activity has been proposed as a mechanism of both carcinogenesis and chemotherapy resistance [141].

Conversely, PKCε acts as an oncogene with antiapoptotic effects when it is overexpressed in cancer cells and in normal rat fibroblasts [142]. PKCε is also oncogenic in colon epithelial cells by interacting with Ras signal transduction pathway, where Ras acts upstream of PKCε [143]. In addition, PI3K is involved in PKCε-mediated oncogenic signaling in the same cells [144]. PKCε has been shown to be involved in the apoptotic pathways both in cancer research and in cardiology [145,146]. Tumor necrosis factor-related apoptosis inducing legend (TRAIL) is a promising anticancer agent since it selectively kills tumor cells but spares normal ones. In contrast, tumor cells are likely to be resistant to anticancer agents due to resistance to TRAIL. Research on breast cancer MCF-7 cells, has demonstrated that PKCε is a major contributing agent for resistance. Specifically, overexpression of PKCε in MCF-7 cells inhibits activation of caspases 8 and 9 and decreases tumor necrosis factor-induced mitochondrial depolarization, which causes the release of cytochrome *c* and cell death induced by TRAIL [110]. Likewise, the level of the antiapoptotic protein Bcl-2 increases,

whereas that of the apoptotic protein Bid decreases by PKCε at both the protein and mRNA levels.

Using the MCF-7 cells, PKCε was also demonstrated to be involved in intrinsic apoptotic pathway, which is triggered by DNA damage. DNA-dependent protein kinase (DNA-PK), which is fundamentally involved in this pathway, is activated upon DNA damage, and plays an important role in DNA repair [147]. PKCε enhances the interaction between DNA-PK and Akt, resulting in phosphorylation of Akt at Ser^{473}. Thus, PKCε acts upstream of Akt to regulate antiapoptotic signaling in breast cancer cells. In small cell lung cancer cells (SCLC), PKCε also shows an antiapoptotic effect [145]. A high percentage of patients with SCLC die due to chemoresistance. This may be caused by the increased expression of antiapoptotic proteins, X chromosome-linked IAP and Bcl-xL [147]. These effects are mediated through the formation of a specific multiprotein complex involving PKCε, B-Raf and S6K2. S6K1, Raf-1 and other PKC isoforms do not form similar complexes [145].

5.2. Preconditioning and myocardial protection

In the last years, different mechanisms were reported as providing powerful protection against myocardial infarction and limiting the severe sequelae of ischemia/reperfusion [148]. Among them, preconditioning was defined as a process in which low repetitive ischemic insults protect organs and tissues from further severe ischemia–reperfusion. Preconditioning has been recognized in the liver, heart and brain [149] and was as well confirmed in patients with coronary artery disease [150].

It is nowadays worth noticing that the development of preconditioning involves several pathways centered in mitochondrial functions. The integration of the activity of protein kinases, nitric oxide synthesis and activation and opening of mitochondrial ATP-sensitive K^+ ($mitoK_{ATP}$) channels support the platform for cardiac protecting preconditioning [151]. $MitoK_{ATP}$ channels are present in the inner mitochondrial membrane as well as in the plasma membrane; however, the two protein constituents of the channels the sulfonylurea receptor subunit SUR2A and the potassium channel Kir proteins are likewise in different proportion in cell and mitochondrial membranes and even, some reports suggested that Kir proteins are absent from the mitochondrial channels. The ischemic insult induces an increase of Ca^{2+} in cells and mitochondria encompassed with an increase of superoxide anion and diffusible H_2O_2 and, a reduction of electron transfer rate and O_2 uptake and ATP. In the mechanism of preconditioning, the opening of $mitoK_{ATP}$ channels induces an increase of matrix K^+ and osmotic H_2O_2 with an augment of mitochondrial volume (swelling) and a decrease of ADP translocation and membrane potential that limits the abnormal mitochondrial Ca^{2+} uptake and reduces a putative corrective effect on membrane potential by extramitochondrial glycolytic ATP. Moreover, Ca^{2+} increases eNOS and mtNOS activities. A moderate increase of mitochondrial steady state NO concentration increases the mechanical efficiency of myocardium by impeding excessive O_2 utilization during ischemia at very low O_2 availability, and leads to a detectable burst of superoxide anion, particularly released at complex I [152].

PKCε is constitutively expressed in mitochondria and associated with the mitochondrial inner membrane [153] and stimulates $mitoK_{ATP}$ channels. Nitric oxide participates in cardioprotection and was as well recently recognized as stimulator of $mitoK_{ATP}$ channels by itself or indirectly through stimulation of PKCε. The effects of NO and PKCε are potentiated by activation of eNOS and PKCε by Akt, which in turn is synergically activated by double phosphorylation in Ser^{473} and Thr^{308} by mTOR2C and PDK1, respectively in the presence of PI3K. Once the PI3K pathway is activated by ischemia and reperfusion, P-Akt concentration increases and NO and PKC and other substrates remain active avoiding the mitochondrial damage imposed by the next episodes. We previously demonstrated that P-Akt translocates to the mitochondria and activates mtNOS *in situ* [101]. Since matrix NO decreases the electron transfer rate and the production of ATP that inhibits the $mitoK_{ATP}$ channels, it is expected that preconditioning is facilitated by mtNOS with further NO-reduction of membrane potential as well as by a burst of NO-released H_2O_2 by the organelles [50] that in turn contributes to Akt and PKC phosphorylation. Indeed, both NO and H_2O_2 facilitates conformational changes of PKC that opens the phosphorylation site. Kinase phosphorylation is thereby an important step of the signaling conducting to the opening of $mitoK_{ATP}$ channels since it is abolished by supplementation with kinase phosphatases, like PP2A. Otherwise, a positive side-effect of the presence of mitochondrial Akt is the phosphorylation and inactivation of GSK3α/β that contributes to tissue protection [154].

6. Therapeutics: kinases as drug targets

Deregulation of kinase activity has emerged as a major mechanism by which cancer cells evade normal physiological constraints on growth and survival. Current popularity of kinases as drug targets is driven by the convergence of several factors. First, with approximately 518 kinases encoded in the human genome, virtually every signal transduction process is wired through a phosphotransfer cascade, suggesting that inhibition of kinase activity can elicit a real physiological response. Accordingly, inhibition of the tyrosine kinase activity of the oncogenic BCR (breakpoint cluster region)-ABL1 fusion protein, which is a causative transforming event in chronic myeloid leukaemia (CML), could be a validated therapeutic intervention [155,156]. Second, despite a high degree of conservation in the ATP-binding site, highly selective small molecules with favorable pharmaceutical properties can be developed [157]. Third, surprisingly, inhibition of kinase activity in normal cells can often be tolerated, presenting a therapeutic window for the selective killing of tumor cells.

The MAPK cascade can be targeted at diverse levels, ranging from receptors in the plasma membrane through Ras, Raf and MEK to ERK. A number of compounds, such as the tyrosine kinase inhibitor, Imatinib, which inhibits the ABL, KIT and PDGFR kinases [158], have been developed to inhibit receptor function. Focusing on the intracellular components of the MAPK cascade, farnesyl transferase inhibitors (FTIs) show positive responses for breast, pancreatic and colorectal cancers as well as leukaemia. These compounds compete with farnesyl transferases for the CAAX motif, preventing the addition of a farnesyl group. By this means, FTIs inhibit membrane localization of Ras [159]. Although FTIs have evidenced some success in treating hematologic cancers, their use as Ras inhibitors has been complicated by the fact that K-Ras and N-Ras (the Ras isoforms commonly mutated in cancer) undergo alternative prenylation by a related enzyme, geranylgeranyltransferase I (GGTaseI), when FTase activity is inhibited by FTIs (Fig. 4). Hence, FTIs are not effective inhibitors of Ras function. Nevertheless, although FTIs are no longer considered Ras inhibitors (except for H-Ras), they have continued to progress through preclinical and clinical development, and preclinical studies are focused on identifying the true targets that contribute to the antitumor activities of FTIs. Raf kinases also supply attractive pharmacotherapeutic targets, and several C-Raf inhibitors are currently in clinical trials. ISIS5132 is a small antisense oligonucleotide that reduces C-Raf mRNA and kinase activity in metastatic breast cancer and metastatic ovarian cancer. Small molecule inhibitors of C-Raf, have also evolved into early clinical trials [160]. The multi-kinase inhibitor, sorafenib, which targets B-Raf, C-Raf, VEGFR and PDGFR, has been approved by the US Food and Drug Administration in 2005 for the treatment of advanced renal cell carcinoma in adults. Downstream from B-Raf, antagonism of MEK is being studied as potential therapy for melanoma. Two MEK inhibitors, PD0325901 and AZD6244, attenuate *in vitro* proliferation, soft-agar colony formation and matrigel invasion of melanoma cell lines with the V600EB-Raf mutation [161]. Interestingly, melanoma cells with V600E B-Raf are more sensitive to MEK inhibition than melanoma cells harbouring an activating Ras mutation [162],

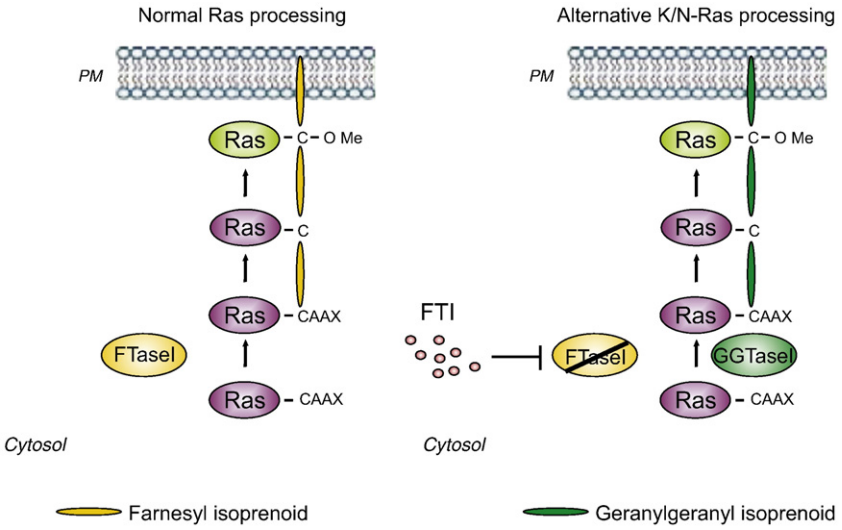

Fig. 4. Post-translational processing of Ras. Normal Ras processing: under normal physiological conditions Ras undergoes three CAAX-motif signaled post-translational modifications catalyzed by the cytosolic farnesyltransferase (FTase), which catalyzes covalent addition of the C15 farnesyl isoprenoid. Alternative K/N-Ras processing: when FTase activity is blocked by farnesyltransferase inhibitors (FTIs), N-Ras and K-Ras (the Ras isoforms most commonly mutated in cancer) will undergo alternative prenylation by the related geranylgeranyltransferase I (GGTase) enzyme, which adds the more hydrophobic C20 geranylgeranyl isoprenoid to the cysteine residue of the CAAX motif. As alternatively prenylated Ras retains normal membrane association and function, FTIs fail to block the function of these Ras isoforms. H-Ras does not undergo alternative prenylation, and consequently, its function is effectively blocked by FTI treatment.

suggesting mutant Ras signals through pathways other than MEK/ERK in melanoma.

As described above, MAPK compartmentalization and further signaling determine the cellular response to the stimulus. Therefore, it would be advantageous to inhibit MAPK in a specific cellular domain, such as the nucleus or mitochondria, while minimising interference of MAPK signaling from other parts of the cell. Essentially, by not inhibiting total cellular MAPKs, pathways that are not involved in the disease or disorder should be spared. Such therapies could provide high selectivity with potentially fewer adverse effects. Although conceptually appealing, a greater understanding of MAPK signaling from distinct subcellular compartments is required before such compounds can be developed.

Other strategies have been developed in cancer treatment, including those inhibiting NFκB [163]. Pharmacological induction of oxidative stress and apoptosis by tamoxifen, the anticancer drug, has been ascribed to increased mitochondrial Ca^{2+} and mtNOS activation in rat liver and human breast cancer MCF-7 cells [164]. Moreover, the arachidonic acid metabolite 12(S)-hydroxyeicosatetraenoic acid, which is involved in pathological conditions associated with mitochondrial oxidative stress, induces mtNOS activation and formation of $ONOO^-$ with subsequent cytochrome *c* release and apoptosis [165]. Similarly, the photodynamic therapy with sensitizers results in a significant increase in intracellular singlet oxygen ($^1O_2$) and in mtNOS activation with cytochrome *c* release and apoptotic death, a process that is attenuated by NO and $^1O_2$ scavengers [166].

The abnormal activation of the PI3K/Akt pathway has been validated as an essential step towards the initiation and maintenance of human tumors. Constitutive pathway activation can result from the distinct and/or complementary biological events including: (i) constitutively active mutants or amplification of receptor tyrosine kinases (epidermal growth factor receptor or ErbB2) leading to constitutive recruitment and activation of PI3K and downstream effectors; (ii) amplification of PI3K; (iii) presence of activating mutations in the PI3KCA gene encoding the p110α catalytic subunit; (iv) overexpression of the downstream kinase Akt; and (v) loss or inactivating mutations of the tumor suppressor gene PTEN, an endogenous negative regulator of the PI3K pathway. The preceding alterations trigger a cascade of biological events, from cell growth and proliferation to survival and migration, which drive tumor progression. In addition, for these survival and proliferation functions, PI3K is a key regulator of angiogenic pathways and up-regulated metabolic activity in tumors. Finally, this pathway also plays a major part in the resistance of tumor cells to conventional cytotoxic and targeted anticancer therapies [167].

Two well-known and isoform-unselective PI3K inhibitors are the fungal metabolite wortmannin [168] and LY294002 [169]. These compounds block the enzymatic activity of PI3Ks by different mechanisms. Wortmannin is an irreversible inhibitor that forms a covalent bond with a conserved lysine residue involved in the phosphate-binding reaction [170], whereas LY294002 is a classical reversible, ATP competitive PI3K modulator [171]. Both compounds have served as important research tools for more than a decade in elucidating the role of PI3Ks in the biology of human cancers. In parallel with their preclinical use, the scaffolds of wortmannin and LY294002 have been the subjects of intense lead optimization efforts directed at improving upon the pharmaceutical limitations of the parent compounds. Thus, using wortmannin as a starting point, broad-spectrum PI3K inhibitors will improve pharmaceutical properties and therapeutic indexes. PWT-458 is a PEGylated derivative of wortmannin that has a higher therapeutic index in preclinical animal models compared to the parent compound. Upon intravenous (i.v.) administration, the polyethylene-glycol (PEG) moiety is cleaved and 17-hydroxywortmannin is released. *In vivo* antitumor activity has been reported in several human xenograft models (for example, U87MG, A549 and A498). PX-866 is a semisynthetic viridin derivative that potently and irreversibly inhibits PI3Kα, γ and δ. Unlike wortmannin, it shows a certain level of selectivity over PI3Kβ and higher stability [171]. These wortmannin derivatives have not entered clinical trials at this time.

Similar efforts have been used to improve the pharmacological properties of LY29002 and derivatives. SF-1126 [172] is a water soluble prodrug of LY294002. The compound, which is designed to target specific integrins within the tumor compartment by means of an appended peptide moiety, inhibits all paralogs of PI3K and other members of the PI3K family, including DNA-PK and mTOR. It blocks the phosphorylation and activation of Akt in cellular settings with IC_{50}

values in the low micromolar range. This prodrug approach appears to enhance the delivery of the active PI3K inhibitor to tumors resulting in significant antitumor activity in xenograft models of brain, neuroblastoma, non-small cell lung, prostate, myeloma, renal and colon carcinoma. In addition to its direct activity on cancer cells, microvessel density analysis of tumor tissue demonstrated that the compound also had significant antiangiogenic activity *in vivo*. The safety of SF-1126 in humans is currently being tested in phase I trials in which patients are being treated twice per week by i.v. infusion over 1.5 h in treatment cycles of 4 weeks. In parallel with the synthetic efforts made to overcome the pharmaceutical hurdles encountered with wortmannin and LY294002, high throughput screening and medicinal chemistry activities have been extensively utilized to discover and develop new selective PI3K modulators. Overall, the new generation of PI3K inhibitors has improved selectivity and drug like properties when compared with the earlier compounds. NVP-BEZ235 is an imidazo[4,5-c]quinoline derivative that inhibits PI3K and mTOR kinase activity by binding to the ATP-binding cleft of these enzymes [173]. The compound potently inhibits proliferation in a broad panel of tumor cell lines by specifically blocking the biological function of PI3K signaling components and inducing a G1 arrest. The antiproliferative activity of this imidazoquinoline derivative in cellular settings translates well in *in vivo* models of human cancer. Thus, compound treatment results in disease stasis or tumor regression when administered orally – 25 to 50 mg/kg/day – and enhanced the efficacy when used in *in vivo* combination studies (for example, docetaxel or temozolomide).

Given their many cellular roles, PKC [174] isozymes are undoubtedly attractive targets for therapeutic intervention. However, the very factors that make them attractive targets also pose significant problems in designing an inhibitory strategy. The complexity of their interactions and the many secondary messenger systems involved coupled with their cellular and tissue-specific variability, renders selective drug action difficult. Staurosporine, identified 20 years ago as an antiproliferative agent, is a potent inhibitor of PKC. It probably acts as a competitive inhibitor by binding to the conserved ATP-binding sites on PKC. Although its specificity for PKC and its isozymes is poor, staurosporine has served as a lead compound from which many other PKC inhibitors have been developed [175]. Midostaurin (also known as PKC412 or *n*-benzoylstaurosporine), similar to UCN01 (7-hydroxystaurosporine), exhibits improved selectivity for PKC ATP-binding sites, but shows modest isozyme specificity. In addition to nPKCs and cPKCs, midostaurin inhibits other tyrosine kinase pathways, including VEGFR2, fms-related tyrosine kinase 3, platelet-derived growth factor receptor and KIT kinases [176]. In preclinical studies midostaurin showed a broad range of antitumor activity, synergizing with conventional cytotoxic agents without overt toxicity and reversing p-glycoprotein-mediated multidrug resistance *in vitro* and *in vivo* [177]. The bryostatins are a family of at least 20 naturally occurring macrocyclic lactones derived from the marine bryozoan *Bugula neritina* [178]. Bryostatin 1 is a potent modulator of PKC activation [179]. Short-term exposure of tumor cells to bryostatin 1 results in cPKC and nPKC activation and translocation to the nuclear membrane [180]. Conversely, prolonged exposure results in membrane depletion of PKC and decreased PKC activity [181]. In addition, bryostatin 1 down-regulates some PKC isozymes, notably PKCα, through the proteasomal degradation of the enzyme [182]. In preclinical models bryostatin 1 inhibits cell growth, angiogenesis and promotes differentiation and apoptosis. The basis for the divergent activities of bryostatin 1 was derived from differential isoform activation (PKCs α, δ and ε) [183] or nuclear translocation (PKCβ), but whether the same mechanisms are active in every tumor type is not clear. In order to achieve greater isozyme specificity, antisense oligonucleotides or peptide fragments have been developed to either inhibit or promote the translocation of PKC isoforms to specific anchoring proteins [184].

Kinase signaling cascades involve an intricate array of interconnected circuits. This complexity suggests that targeting this pathway will be not as straightforward as once imagined. Additionally, as the clinical analyses of these inhibitors progress (Table 2), developing a greater appreciation of the complex biology and genetics of the cancer cell will be crucial in understanding how clinical trial design can be improved.

7. Concluding remarks

The term *signal transduction* was first coined in 1969 by Martin Rodbell in an attempt to describe how cells receive, process, and ultimately transmit information from external signals, such as hormones, drugs, or even light. Rodbell viewed signal transduction as a process in which receptors on the cell surface receive information from outside the cell and transmit this information to an amplifier or effector. However, this simple model was subsequently revised to incorporate new science showing that many signaling proteins act controlling the amplitude of signaling output with even higher levels of molecular control, to integrate input from multiple cellular stimuli, using functionally important conformational changes in response to cofactors, translocation events, and post-translational modifications (phosphorylation, oxidation, tyrosine nitration, etc.).

Mitochondria are now accepted as important players in apoptotic events. About two billion years ago, after the incorporation of purple eubacteria and the formation of a symbiotic relationship, cells acquired the ability to survive in a new aerobic environment [185]. As a key point in oxidative metabolism in eukaryotes, mitochondria supply the majority of cellular energy, sustaining viability and cell functions. It is now widely established that mitochondria are not exclusively ATP suppliers but take part in regulatory and signaling events, responding to multiple physiological inputs and regulating cell proliferation and death. They sense and integrate signals from translocated proteins and Ca^{2+} fluxes, and transmit them to oxidative stress and other metabolic pathways [186]. Mitochondria have been shown to be dynamic tubular networks that constantly undergo fusion and fission in response to local environmental changes, with associated changes in their morphology. The presented data indicate that mitochondria integrate a three compartment cycle that allows MAPKs and Akt to be posttranslationally oxidized and controls its degree of phosphorylation and in consequence their subcellular movements (Fig. 5A and B). These effects differentially activate cell proliferation through regulation of genes and mitochondrial components while H_2O_2 accumulation gradually favors the transition from growth to quiescence. The mitochondrial contribution to kinase activation opens new fields in the balance between proliferation and apoptosis. Since disruption between proliferation and apoptosis is a cornerstone of tumorigenesis, the understanding of this mechanism suggests new preventive and therapeutic measures in the treatment of cancer, still the most important cause of death in the industrialized world.

Table 2
Cancer therapeutics based on transduction signal pathway inhibitors.

Agent	Target	Trial phase	Tumors
Imatinib	PDGFR	Approved	CML,GIST
ZD1839 (Gefitinib)	EGFR	Approved II–III	NSCLC, breast, esophageal, brain
BAY 43-9006 (Sorafenib)	VEGFR, PDGFR, c-Kit	Approved	Renal
R115577	FTI	III	Leukaemia
PD0325901	MEK	I	NSCLC, breast, colon, pancreatic
NVP-VEZ235	PI3K	I	Solid tumors
SF1126	PI3K	I	NSCLC, colon, prostate, myeloma
Midostaurin	nPKC and cPKC	I	Leukaemia
Bryostatin 1	PKC	II	Leukaemia, melanoma, lung

CML: chronic myeloid leukaemia; GIST: gastrointestinal stromal tumor; NSCLC: non-small-cell lung cancer.

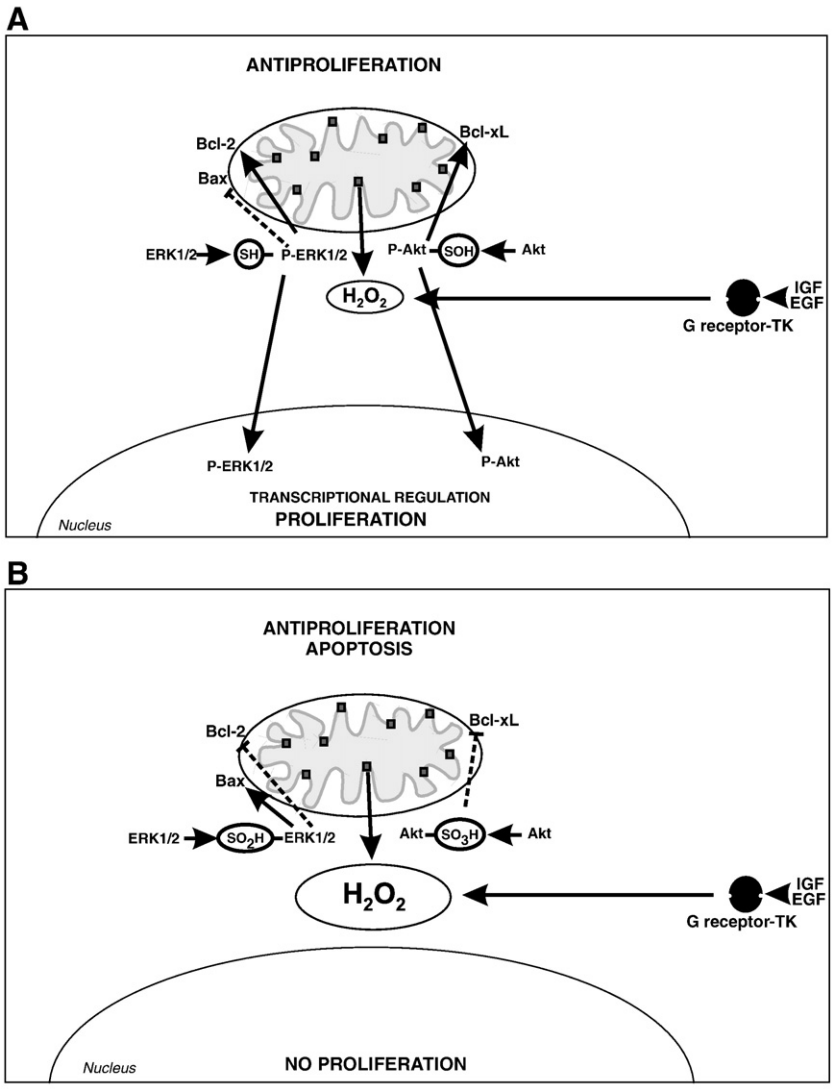

Fig. 5. Growth factors (EGF, IGF) promote a discrete burst of H_2O_2 that does not affect ERK1/2 Cys^{38} and ERK2 Cys^{214} but acts on Akt Cys^{60} and Cys^{310}. In A, none oxidation (ERK1/2) or mild oxidation to –SOH (Akt) of kinase cysteines at low H_2O_2 favors the transition to the respective phosphorylated kinase status that stimulates the translocation to nucleus and transcription of proliferation genes, the inhibition of proapoptotic factors and the stimulation of antiapoptotic mitochondrial components. In B, high H_2O_2 allows cysteine oxidation to $–SO_2H$ and $–SO_3H$ that impedes kinase phosphorylation, translocation to nucleus and proliferation, and stimulates the proapoptotic mitochondrial machinery. Squares represent the oxidative units of the electron transfer chain.

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